Description of the invention

The main purpose of this invention is to provide a biopolymer, produced by an enzymatic extract or preparation having glucosyltransferase and fructosyltransferase activity. It is produced from a *Lactococcus lactis* strain (NRRL B-30656) characterised by its high transfer activity, allowing the biopolymer to be obtained by a simple production method which is easy to scale-up. Its production comprises the following steps: **Phase 1**: fermentation with the *Lactococcus lactis* NRRL B-30656 strain in a culture medium developed for this microorganism's growth; **Phase 2**: extracellular enzyme recovery trough centrifuging or ultra-filtration; **Phase 3**: biopolymer production trough enzyme reaction using sucrose as substrate and the enzymatic extract or preparation; and **Phase 4**: biopolymer purification trough precipitation with solvents or ultra-filtration followed by drying the product.

Detailed description of the invention

The object of the invention is to produce a polysaccharide contaminants-free pure biopolymer. The biopolymer can be described as being a polymer produced by a *Lactococcus lactis* strain isolated from soil. This strain has high transfer activity, leading to obtaining the biopolymer through a simple process, having a purity greater than 95%.

The microorganism. The *Lactococcus lactis* NRRL B-30656 strain is isolated from soil in the present invention by a selective process using a sucrose-containing medium sucrose as a carbon source in which the microorganisms producing the enzymatic extract or preparation having glucosyltransferase and fructosyltransferase activity, are able to use the substrate and to produce the polymers, giving the colony a mucoid aspect. Microorganisms having these characteristics are selected from this medium and purified trough isolating techniques involving successive dilutions

Component	**Concentration in g/l**
Salts	
K_2HPO_4	7-30
$FeSO_4.\ 7H_2O$	0.01-1
$MgSO_4.\ 7H_2O$	0.01-0.1
$MnSO_4.\ H_2O$	0.001 – 0.1
$CaCl_2.\ 2\ H_2O$	0.001 – 0.01
NaCl	0.01-0.1
Carbon source	
Sucrose	10-40
Nitrogen source	
Yeast extract	7-30

The pH is set to pH 5-9 with HCl. The medium is sterilised at 121°C for 15 minutes.

Fermentation. The pre-inoculums corresponding to 5-20% of the inoculum volume are activated from the pure strain preserve at -70°C in a medium having 20% glycerol; incubation time should not exceed 10-36 hours during which time pre-inoculum purity must be verified. These cultures are done in flasks with stirring , occupying 5-20% total volume; they are incubated at 20-40°C with 100-400 rpm stirring rate in orbital agitators. The number of inoculums necessary is determined by the number and size of the fermenters.

Growth and enzyme production conditions are 20-40°C temperature with stirring at a rate of 100– 400 rpm (depending on the fermentation scale).

Aeration. The fermentation promoting microorganism is aerobic, meaning that the culture had to be aerated with 0.1 –1 volumes of air per medium volume per minute (vvm) and pH is kept between 5 and 9 during fermentation. Culture mediums resulting from this production process have combinations of components in order to obtain final biomass concentration of 10-30 g/l, a wet weight, having 2-6 U/ml transfer activity, this being achieved in 6-24 hours.

Enzyme recovery. Extra-cellular enzyme are collected from fermented culture medium trough centrifuging at 3,000 -10,000 rpm for 15 minutes or by separating the biomass

trough filtration. Enzymatic extract or preparation thus presents 2-6 U/ml glucosyltransferase and fructosyltransferase activity.

Biopolymer production

Enzymatic reaction. Reaction conditions were as follows:

Reaction medium:

50-300 Mm phosphate buffer pH	: 5-9
Substrate	: 5-40% sucrosa
Quantity of enzyme	: 10-40% v/v enzymatic extract or preparation
Reaction time	: 12-48 hours
Agitation	: 100-400 rpm

Biopolymer recuperation and purification

After the enzymatic reaction, the temperature was reduced to 4°C following enzymatic reaction and the biopolymer was recovered in two ways:

a) Precipitation with solvents

96% ethanol was added to the cold reaction mixture with agitation. The added amount of ethanol corresponds to 1.2- 2.0 volumes of ethanol/ reaction mixture volume.

Example 2

Production of the enzymatic extract or preparation

1. Fermentation:

a) Microorganism activation

The *Lactoccoccus lactis* NRRL B-30656 microorganism was used for obtaining an enzymatic extract or preparation having glucosyltransferase and fructosyltransferase activity. Bacteria were stored in a cryoprotection solution (glycerol) at -70°C. The strain was slowly unfrozen until room temperature was reached and it was activated in 50 ml sucrose medium at 30°C for 12 hours and stirring at 180 rpm. 5 ml of this culture were used for two types of seeding. The first in agar succhrose, incubated at 30°C for 24 hours, while observing its mucoid characteristics and then stored at 4°C; the second in 100 ml sucrose broth incubated at 30°C for 12 hours. The latter was distributed in 1 ml centrifuge tubes with 20% v/v glycerol and stored at –70°C, for later use in fermentations. The remaining 45 ml of initial culture were preserved in 5 ml vials, lyophilised using 10% concentration sterile skimmed milk as support and stored at 4°C.

b) Preparing pre-inoculums and inoculums

Pre-inoculums were prepared with the same medium composition corresponding to the batch; the microorganism conserved in solid sucrose medium was taken, then seeded in a volume of liquid medium, at 5-20% inoculum volume, cultured at 25-35 °C, with stirring at 100-400 rpm for 12-24 hours.

Composition of the medium used:

Component	g/l concentration
Salts:	

Fermenter operating conditions

Conditions	14 l
Medium volume (l)	10
Medium volume/fermenter volume ratio	0.8
Inoculum percentage	5-10
Inoculation optical density	0.5-1
Stirring (rpm)	100-400
Temperature (°C)	25-35
Aeration (vvm)	1-3
Initial medium pH	5-8

Fermentation time (hours)	6-12

2. Enzyme recovery:

a) Centrifuging

Extracellular enzyme was recovered by centrifuging at 5,000 rpm for 15 minutes for separating the biomass. The enzymatic extract or preparation presented 2–6 U/ml glucosyltransferase and fructosyltransferase activity.

b) Ultrafiltration

Another way of recovering fermentation supernatant is by using 0.22-2 micra pore size ultra filtration membranes.

Example 3

Biopolymer production and recovery

a) Enzymatic reaction. Reaction conditions were as follows:

Reaction medium:

50-200 Mm phosphate buffer pH : 5 - 7

Substrate : 8-20% sucrose

Enzyme quantity : 10-30% v/v enzymatic extract (200-500 U/l).

Reaction time : 20-40 hours

Stirring : 100-400 rpm

The enzyme was separated by centrifuging, placed in medium containing 8-20% sucrose, at pH 5-8 and 25-35°C for 20-30 hours, obtaining 30-60 g/l polymer concentration corresponding to 40-60% yield regarding substrate. Other processes reported to date have required up to 5-10 days for producing polymer. The reported microorganisms produced less poly mer concentration (See Table 1).

b) Purifying the biopolymer

After the enzymatic reaction, the temperature was lowered to 4°C following enzyme reaction and it was possible to recover the biopolymer in two ways:

- **Precipitation with solvents**. 96% ethanol was added to cold reaction mixture with stirring. The quantity of added ethanol corresponded to 1.0-3.0 volumes of ethanol/volume reaction mixture.
- The precipitated biopolymer was dissolved in half the volume of deionised and distilled water and precipitated again with 1.0 to 3.0 volumes of ethanol/ reaction mixture volume.
- Precipitated biopolymer was redissolved in third of the volume of water and dried by lyophilisation or dried by compressed air at 60-80°C until reaching 5-10% humidity.

Table 1

EPS production using different microorganisms

Organism	Biopolymer (g/100 ml)

c) Drying

The final product was obtained as a white powder that can which could be dried by lyophilisation or dry heat at a temperature not greater than 80°C.

Example 4

Biopolymer characterisation

1. Solubility

The product was a hydro-soluble biopolymer able to form hydrogel homogeneous dispersions up to 50% maximum concentration. 1.0 g of biopolymer was dissolved in 32 ml 5% chlorhydric acid, in 50 ml 10% sodium hydroxide in 30 ml glacial acetic acid.

It was insoluble in ethanol, isopropanol, acetone, mineral and vegetal oil and polyethylenglycol.

The product was moderate soluble in 0.5% oxalic acid at ebullition temperature.

2. High performance liquid chromatography (HPLC).

- A 1.5% biopolymer solution presented a 900-1,100 KDa molecular weight in permeation chromatography determined on a Shodex OHPak KB-803 column. Chromatography conditions were as follows:

Temperature	: 55°C
Mobile phase	: 0.1 M NaCl solution
Flow	: 0.9 ml/min

- Polymer purity was greater than 95%, shown by a thin peak in HPLC, under the following conditions:

Column:	Shodex SC1011
Mobile phase:	distilled deionised water
Flow:	0.6 ml/min.
Temperature:	70°C.
Equipment:	Waters 510 with refraction index detector (Waters 2410).

The biopolymer presented a 7 to 7.5 minute retention time under these conditions.

The patterns used were analytic reagent grade glucose, fructose, and sucrose .

- The biopolymer was stable over broad range of pH shown by HPLC after contacting the polymer with pH 2-9 buffers.

3. Viscosity

Viscosity was determined in a 10% solution at 30°C using a ViscoEasy viscosimeter Serie L, Schott, Ref. 28.541.120, L2 stem at 50 rpm. The samples analysed presented viscosity ranging from 1,000-3,000 centipoises (cP). Pseudo-plastic behaviour was exhibited (cross-sectional thinning). Biopolymer solution viscosity became reduced on increasing the shear rate and increased on reducing temperature.

4. Dimensional characteristics

The biopolymer had a true density close to that of sucrose (1.5 mg/ml). It is a material presenting high inter-particle porosity (48%).

Average DVS particle size (diameter/volume/surface) was 224 micron.

5. Humidity adsorption

Water adsorption capacity ranged from 6.12 mg/g to 353.20 mg/g depending on relative humidity; this means that it was a slightly hygroscopic material. The biopolymer was capable of unlimited expansion on contact with water due to its polymeric structure and hydrophilicity, being able to form variable consistency systems depending on the quantity of water incorporated, giving rise to forming aqueous dispersions characterised by their high viscosity.

6. Humidity

It presented losses of up to 10% when dried in a vacuum oven at 60°C.

7. Thermal characteristics

The biopolymer presented two vitreous transition points; the first between 20°C and 30°C and the second between 190°C and 220°C as determined by differential scanning calorimetry.

8. Microbiological quality

The biopolymer presents the following microbiological counts:

Microbiological charge	Range	Unit
Viable mesophile count	2000 - 4000	cfu / gr
Coliform count	Absence	mpn / gr
Faecal coliform count	<10	mpn / gr
Salmonella count	Absence	
Mould and yeast count	2000 - 5000	cfu / gr

9. Uses

The biopolymer could be used in the pharmaceutical industry as viscosant, thickener, stabiliser, dispersant, as a film former,